FINAL REPORT FOR

"A STUDY TO ASSESS THE EFFECTS OF THROMBIN ON BONE REGENERATION"

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Thrombin is occasionally used as a hemostatic agent during bone surgery in humans and animals. Recent evidence from studies in vitro has indicated that thrombin stimulates bone resorption. The effect of 100 units thrombin on bone wound healing and bone resorption in rats was tested in untreated 8-mm circular craniotomy defects treated with 25 mg demineralized rat long bone matrix or 25 mg demineralized calvarial bone matrix. Twentyeight days after treatment, defects were recovered and radiographed. As an indicator of bone, radiopacity within the defect area was quantified using computarized image analysis. In the absence of thrombin, bone resorption occurred in untreated defects(5.3 ± 5.1 mm^2) and defects treated with calvarial bone matrix(6.8 ± 5.1 mm^2); the use of thrombin had no additional effect on resorption in these groups. Long bone matrix prevented bone resorption and significantly stimulated bone formation by 77%, compared with untreated controls, and by 73%, compared with defects treated with calvarial bone matrix. Addition of thrombin to long bone matrix diminished the inductive response by 14%, though the amountof bone formed in the presence of thrombin

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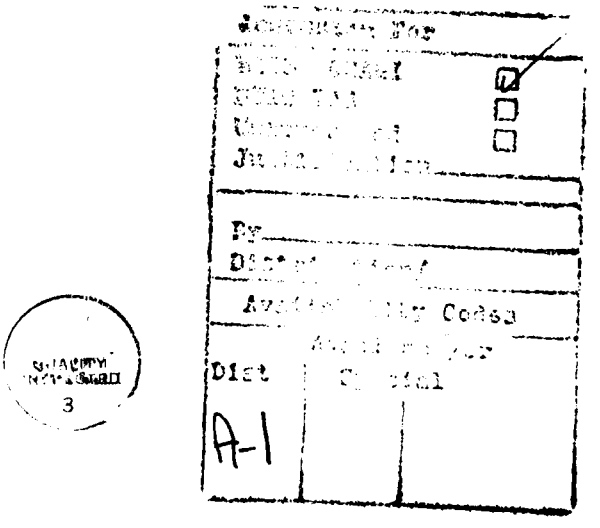
was still significantly greater than in untreated control defects. However, thrombin negated the protective effect of long bone matrix on bone resorption. These data indicate that use of thrombin as a hemostatic agent in bone surgery may be counterproductive to bone healing.

ABSTRACT

Thrombin is occasionally used as a hemostatic agent during bone surgery in humans and animals. Recent evidence from studies *in vitro* has indicated that thrombin stimulates bone resorption. The effect of 100 units thrombin on bone wound healing and bone resorption in rats was tested in untreated 8-mm circular craniotomy defects and defects treated with 25 mg demineralized rat long bone matrix or 25 mg demineralized calvarial bone matrix. Twentyeight days after treatment, defects were recovered and radiographed. As an indicator of bone, radiopacity within the defect area was quantified using computerized image analysis. In the absence of thrombin, bone resorption occurred in untreated defects (5.3 ± 5.1 mm^2) and defects treated with calvarial bone matrix (6.8 ± 5.1 mm^2); the use of thrombin had no additional effect on resorption in these groups. Long bone matrix prevented bone resorption and significantly stimulated bone formation by 77 %, compared with untreated controls, and by 73%, compared with defects treated with calvarial bone matrix. Addition of thrombin to long bone matrix diminished the inductive response by 14%, though the amount of bone formed in the presence of thrombin was still significantly greater than in untreated control defects. However, thrombin negated the protective effect of long bone matrix on bone resorption. These data indicate that use of thrombin as a hemostatic agent in bone surgery may be counterproductive to bone healing.



INTRODUCTION

Suspicion that thrombin may be a bone-resorptive agent arose in 1983 when Gustafson and Lerner (Gustafson and Lerner 1983) searched for an "osteoclast-activating factor" as the causitive agent for bone resorption seen in patients suffering from chronic inflammation. Thrombin (0.1 - 7 units/ml) caused mobilization of calcium and [^{3}H]-proline from cultured neonatal mouse calvariae. Since the effect was reduced in the presence of indomethacin, an inhibitor of prostaglandin (PG) synthesis, the authors suggested the effect of thrombin was mediated through stimulation of synthesis of PGs, potent stimulators of bone resporption (Yamaguchi et al. 1988). They later showed that thrombin stimulated bone resorption even in the presence of inhibitors of PG synthesis, indicating two pathways, one PG-dependent and the other PG-independent (Lerner and Gustafson 1988). These investigators also observed that the resorptive effect of thrombin was lower in fetal rat long bones than in mouse calvariae.

Thrombin is occasionally used in bone surgery in humans and in animals as a hemostatic agent (Tidrick et al. 1943, Codben et al.1976, Harris et al. 1978). It is a major component of fibrin sealant, produced and used in Europe. Investigators have found no deleterious effect on bone healing when the sealant was used either alone (Schlag and Redi 1988) or in combination with demineralized bone matrix or bone matrix gelatin (Schwartz et al. 1989).

This study was designed to show whether application of a thrombin solution to untreated rat critical-size craniotomy defects or defects treated with DBM from calvariae or long bone would inhibit bone regeneration.

MATERIALS AND METHODS

Calvariae and long bones were obtained from 8-week-old male Long-Evans rats. Marrow was washed from bones with sterile water. Bones were defatted with ethanol and ether and ground in a Wiley mill. Bone powder, 72 to 420 μ particle size, was demineralized with 0.5 N HCl, washed with deionized water, dehydrated with absolute ethanol, ether, and dried.

Five thousand units of sterile, freeze-dried thrombin (Thrombostat, Parke-Davis, White Plains, NJ) was dissolved in 5.0 ml isotonic saline containing 5 mg calcium chloride, 8 mg sodium chloride, and 39 mg glycine (vehicle solution), to make a solution containing 1000 units/ml.

Four-week-old Long Evans rats, males and females, were randomly divided into six groups of 18 rats. Animals were anaesthetized by intramuscular injection of a solution containing ketamine (Vetalar), 62.5 mg/ml, and xylazine (Rompum), 6.25 mg/ml, in physiological saline. A midline incision was made from the external occipital protuberance to the nasal bones. The periosteum overlying the frontal, parietal, and occipital bones was scraped away with a scalpel. An 8-mm trephine circular midsagittal defect was made.

The defects were treated as follows:

Group 1: 0.1 ml vehicle solution

Group 2: 0.1 ml thrombin solution

Goup 3: 25 mg Calvarial DBM, 0.1 ml vehicle solution

Group 4: 25 mg Calvarial DBM, 0.1 ml thrombin soution

Group 5: 25 mg Long bone DBM, 0.1 ml vehicle solution

Group 6: 25 mg Long bone DBM, 0.1 ml thrombin solution

DBM was packed loosely into the defect. Vehicle or thrombin solution was applied directly to the exposed defect in groups 1 and 2, or onto the surface of the DBM in groups 3 though 6. Wounds were closed with surgical staples.

Defect sites were harvested from animals euthanized by carbon dioxide asphyxiation 28 days after treatment. An anterior-posterior skin incision exposed the surgical site and surrounding bone. The frontal-occipital-parietal complex was removed and placed immediately into 70% ethanol. When retrieval of the entire defect was not obtained, the speciman was discarded.

Explants were x-rayed (Minishot, TFI Corporation, New Haven, CT) at 3 milliamperes for 10 seconds at 15 kilovolts. Radiopacity inside an 8-mm circle overlaying the original defect was quantified by computerized image analysis (Quantimet 520, Cambridge Instruments, Chicago, IL). Resorption at the edges of the defect was estimated by measuring the area of radiotransparency between the host bone and the area contained by the 8-mm circle.

Data were analyzed using a 2 X 3 factorial analysis of variance design (Abacus Concepts 1987)). Significance was established at $p < 0.05$. Differences between treatment means were determined using Fisher's protected least significance differene (PLSD) test.

RESULTS

Means of each treatment group are graphed in Figures 1A and 1B. Analysis of variance of radiopacity, Table 1, indicated that thrombin had no significant effect on the radiopacity of healing defects, indicating no effect on bone formation. Treatment of defects with DBM, however, affected radiopacity significantly; treatment with long bone DBM resulted in a significantly greater area of radiopacity within the defects than no treatment or treatment with calvarial DBM.

Table 1. Radiopacity

Anova table for a 2-factor analysis of variance of radiopacity using a 2 X 3 factorial design.

Source:	df	Sum of Squares	Mean Square	F-test	P value
Thrombin	1	46.93	46.93	1.276	.2618
DBM	2	772.49	386.24	10.50	.0001*
Interaction	2	56.45	28.23	0.77	.4674
Error	79	3052.14	36.77		

* Indicates a statistically significant effect.

DBM also had a significant effect on resorption whereas thrombin had no significant effect (Table 2). However, the ANOVA indicates a significant interaction between DBM and thrombin; there was a significant difference between resorption resulting from treatment with long bone DBM alone and that with long bone plus thrombin. The fact that this effect was not seen in the untreated group or the group treated with calvarial DBM, suggests a antagonistic effect between long bone DBM and thrombin.

Table 2. Resorption

Anova table for a 2-factor analysis of variance on resorption using a 2 X 3 factorial design.

Source:	df	Sum of Squares	Mean Square	F-test	P value
Thrombin	1	39.81	39.81	2.092	0.1521
DBM	2	150.02	75.01	3.94	0.0234*
Interaction	2	132.81	66.40	3.49	0.0353*
Error	79	1503.40	19.03		

*** Indicates a statistically significant effect.**

In order to get a better idea of the total effect of thrombin or DBM on the calvarial defects, we subtracted the area of resorption outside the original defect area from the area of radiopacity within the 8-mm circle. Analysis of variance indicated only a significant effect by treatment with DBM. There was no interaction between the treatment with DBM and the thrombin. Means of each treatment group are graphed in Figure 1C. Analysis of differences between treatment means showed that long bone DBM elicited a significantly greater total effect than the other two treatments. Thrombin significantly attenuated this effect, but did not completely abolish it.

Table 3. Total positive effect.

Anova table for a 2-factor analysis of variance on total positive effect using a 2 X 3 factorial design.

Source:	df	Sum of Squares	Mean Square	F-test	P value
Thrombin	1	224.54	224.54	2.477	.1195
DBM	2	1652.85	826.42	9.117	.0003*
Interaction	2	328.86	164.43	1.814	.1697
Error	79	7161.37	90.65		

* Indicates a statistically significant effect.

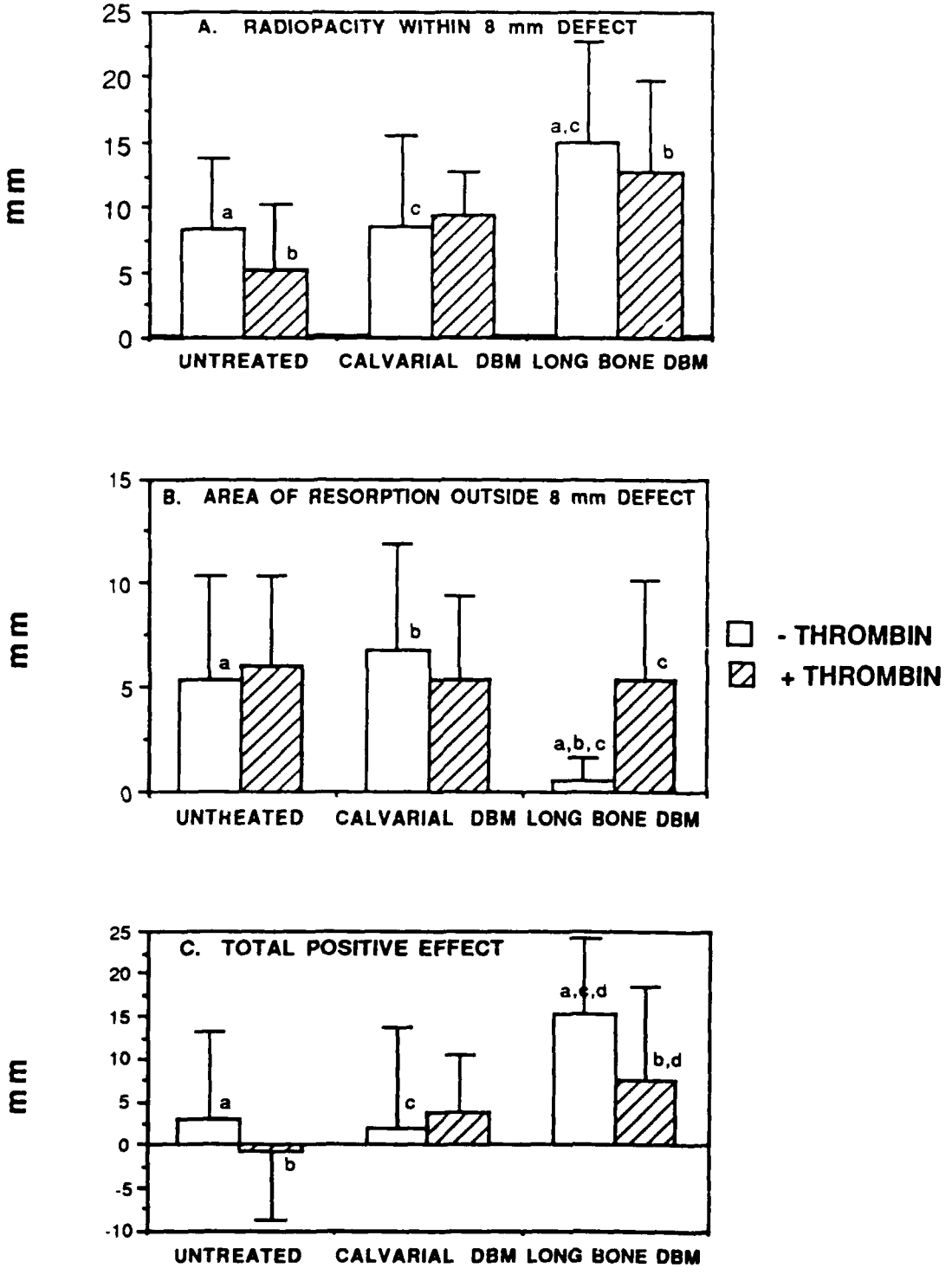


Figure 1. Effects of thrombin and DBM on healing of 8-mm critical-size rat craniotomy defect: areas of radiopacity (A), resorption (B), and combined total positive effect (C). a, b, c, d indicate differences between these pairs of treatment means at $p<0.05$.

DISCUSSION

The use of hemostatic agents in orthopedic surgery has obvious benefits. However, indiscriminate use of such agents, without considering potential harmful side effects, may be counterproductive. Use of thrombin in orthopedic surgery would be contraindicated if thrombin, a potential bone resorptive agent, were inhibitory to bone healing.

The study reported here indicates that in its intended usage, exogenous thrombin did not effect bone repair in critical-size calvarial defects in general, and it did not stimulate bone resorption. There was marked resorption of the defect margins in untreated defects and defects treated with calvarial DBM; thrombin had no additional effect on this resorption. Treatment with long bone DBM resulted in a decrease of bone resorption around the defect edges. This was probably secondary to the bone inductive activity of the DBM. However, thrombin obliterated the anti-resorptive effect of long bone DBM. The effect of thrombin in this instance is puzzling. If thrombin were directly inhibiting bone regeneration, or stimulating bone resorption, one would expect the effect to be general. However, the effect of thrombin was limited to the defect margins. This implies that thrombin induces a secondary effect mediated through cells which arise from the defect margins.

Long bone DBM was the only treatment which significantly stimulated bone regeneration inside the area of the 8-mm defect. Long bone DBM induces bone regeneration by transforming pluripotential mesenchymal cells into bone forming osteoblasts (Reddi et al. 1977). These cells arise from the marrow space of injured bone and from the periosteum and endosteum (Richany et al. 1964). Regeneration of bone is often preceeded by a temporary phase of resorption. It is presumed that this initial resorption is required to liberate bone inducing factors such as osteogenin (Sampath et al. 1987) and bone morphogenetic protein (Urist

et al. 1983), and growth factors, such as basic fibroblastic growth factor (Hauschka et al. 1986, Rogelj et al. 1989) and transforming growth factor (Carrington et al. 1988) from extracellular matrix. These factors could act upon mesenchymal cells, converting them to osteocompetent cells needed for osteogenesis. The fact that untreated calvarial defects and defects treated with calvarial DBM had limited bone regenerative effects probably stems from the fact that mature calvarial bone lacks sufficient reserves of osteoinductive factors (Reddi 1975a, Reddi 1975b, Prolo et al. 1984).

Long bone, on the other hand is the best source for inductive and growth factors (Reddi 1975a, Reddi 1975b). It has the ability to transform preosteoblasts arising from the marrow space and the periosteum of the dura. It is conceivable that thrombin inhibited transformation of mesenchymal cells arising from the marrow space to osteogenic cells. The simplest mechanism for the inhibition of transformation is the physical barrier created by the action of thrombin on blood-bourne fibrinogen in the first step of the clotting cascade. The resulting clot could prevent migration of pluripotential cells from the marrow space into the defect area where they could be acted upon by inductive proteins present in DBM. However, the clot is not permanent and its eventual dissolution would remove this physical barrier. Providing the clot were dissolved within 5 days, the approximate halflife of the bone inductive capacity of DBM (Landesman and Reddi 1989), a normal progression of cells into the defect area could still occur, given the chemoattractive nature of DBM (Landesman, 1986).

If thrombin were inhibiting progression or transformation of preosteoblasts from host marrow, why not from periosteum? The simplest explanation stems from the application of thrombin. Thrombin was applied to defects after application of long bone DBM. The DBM probably shielded the dura, causing thrombin to accumulate in the space between DBM and host bone. Further experiments using detailed histological examination would be required to

elucidate the mechanism of thrombin.

The evidence presented by previous investigators concerning the possible mechanism of thrombin's actions is not consistent with the data presented here. We saw no increased rate of resorption following treatment with thrombin, only an inhibition of bone regeneration. The disparity of effects of thrombin on bone seen in cases of chronic inflammation or in tissue culture and those reported here or with the use of fibrin sealant could indicate the difference between chronic and acute activity. Lerner and Gustafson reported a lag period of 12 to 24 hours of continuous treatment before the bone-resorptive effect of thrombin *in vitro* was detectable (Lerner and Gustafson 1988). It is doubtful that thrombin *in vivo* would remain active this long; *in vitro*, thrombin has a short half-life and probably loses its activity within three hours (Physicians' Desk Reference, 1990). We used a dose of thrombin 10 times that recommended for general surgery. Perhaps a greater effect would have been seen after treatment of CSDs with solid thrombin.

The amount of radiopaque material formed in response to long bone DBM was suboptimal compared with that seen in previous experiments. Frequently, CSDs treated with 25 mg of long bone DBM heal completely within 28 days (Mark et al. 1990). Unfortunately, the bioactivity of the DBM used in this experiment was not assessed in heterotopic sites prior to use in the experiment. The limited bone induction in response to treatment with calvarial DBM was less surprising. Calvarial bone normally has little bone-inducing capacity compared with long bone from the same animal (Reddi 1975a, Reddi 1975b). In order to validate the results of this experiment it would be prudent to test the effects of thrombin on a more active preparation of DBM, and use histomorphometry to quantitate bone formation.

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